Troubleshooting Fundamentals

Paul Altiero
Applications Chemist, Agilent
07 October 2021

DE44473.3563078704

Agenda

Chromatographic indicators

Tools of the trade

Troubleshooting

Benchmarking

DE44473.3563078704

Chromatographic Indicators:

How does our chromatogram tell us that something is wrong?

- Peak area
- Retention time
- Peak shape

Factors that Affect Peak Area

Injection errors

- Needle, needle seat and rotor seal: Clogs or ware can cause loss of volume accuracy.
- Injector settings: Changes to draw speed or needle offset can cause aspiration errors.
- Multiple injections: When injecting from the same vial we need to take extra precautions.

Sample issues

- Sample age: Check stability and concentration from evaporation.
- Solvent and volume issues: When is 2 mL not 2 mL?
- Containment: Check for binding and contamination.

Detector issues

- Detector settings: Optimize peak width and check the background.
- Age and performance: Run detector diagnostics to confirm age and response of lamp.
- PMCS: Is the detector clean and equilibrated?

Factors that Affect Retention Time Precision

- Insufficient equilibration: Most notable in early eluting peaks.
- Change in mobile phase: This can include something as simple as pH changing due to equilibration with atmospheric CO_2.
- Change in flow rate: Pump errors and clogged valves can result in the system delivering less solvent. Run pump diagnostics to confirm good performance.
- Change in column temperature: Improper thermal equilibrated, cover left off TCC, instrument errors.
- Other instrument issues: Has the delay volume changed?

Separation Conditions That Cause Changes in Retention*

Flow rate	**± 1%**	**± 1% Tr**
Temp	**± 1 °C**	**± 1 to 2% Tr**
%Organic	**± 1%**	**± 5 to 10% Tr**
pH	**± 0.01%**	**± 0 to 1% Tr**

*excerpted from “Troubleshooting HPLC Systems”, J. W. Dolan and L. R. Snyder, p 442.

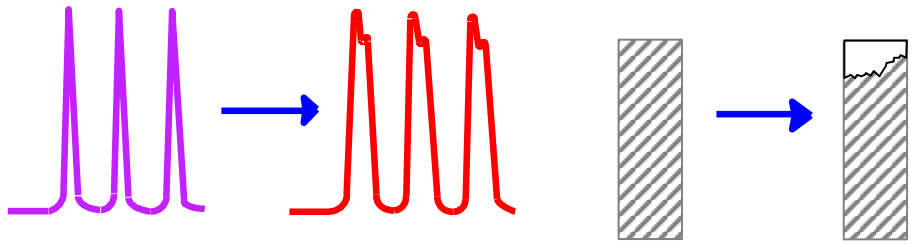
What Are Common Peak Shape Issues?

- Peak tailing/fronting
- Broad peak
- Split peaks

- Many peak shape issues are also combinations, i.e. broad and tailing or tailing with increased retention.
- Symptoms do not necessarily affect all peaks in the chromatogram.
- Each of these problems can have multiple causes.

Peak Splitting Caused by Disrupted Sample Path

- Flow path disrupted by void
- Sample allowed to follow different paths through column
- Poorly packed bed settles in use
- High pH dissolves silica



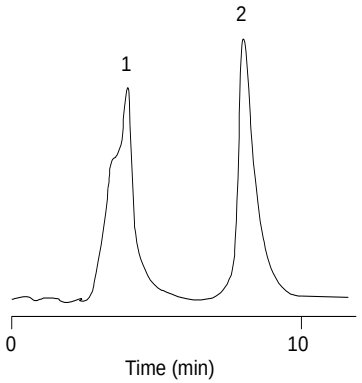
Normal **Double peaks**

Tip: Similar effect can be caused by partially plugged frit.

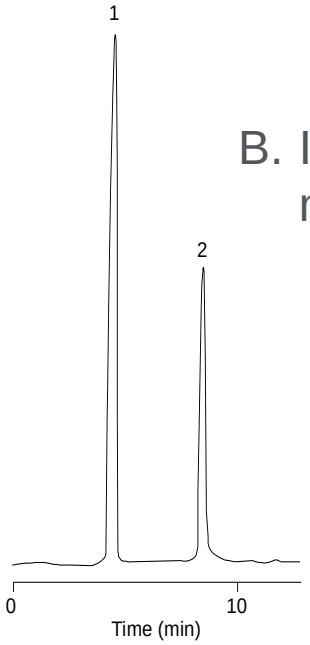
Split Peaks from Injection Solvent Effects

Column: Agilent StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile phase: 82% H_2O:18% ACN
Injection volume: 30 µL Sample: 1. Caffeine 2. Salicylamide

A. Injection solvent 100% acetonitrile



B. Injection solvent mobile phase

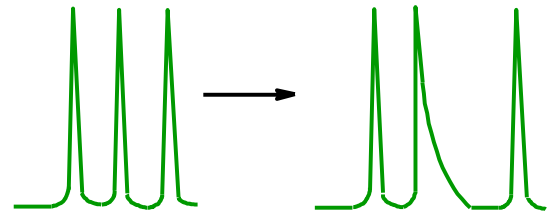


Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening

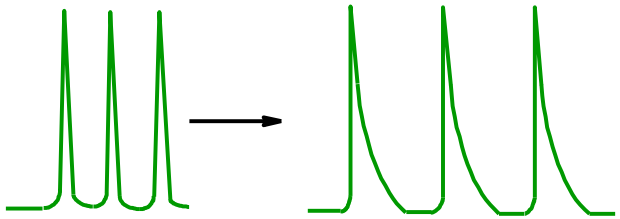
Trick: Keep organic concentration in sample solvent ≤ mobile phase

Peak Shape: Tailing Peaks

Symmetry > 1.2



Normal Tailing

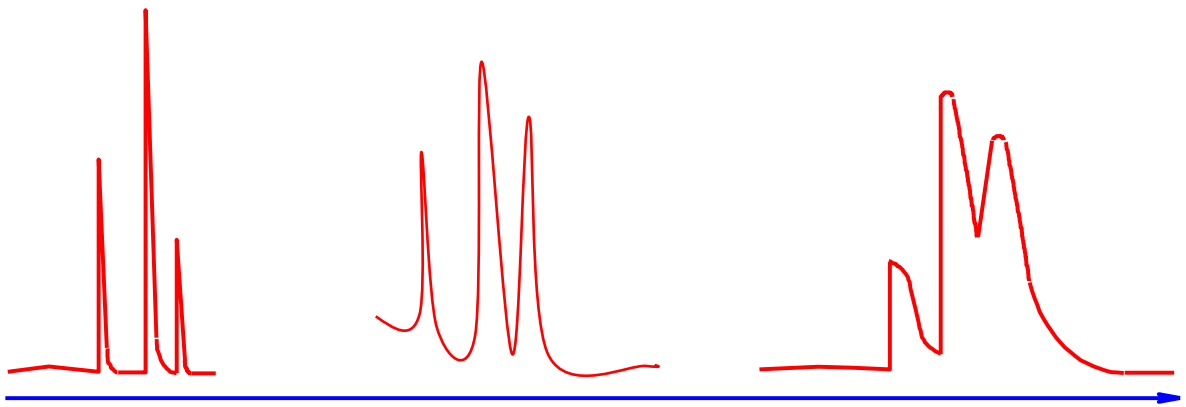


Normal Tailing

Common causes

- Some peaks tail
  - Secondary-retention effects
  - Residual silanol interactions
  - Small peak eluting on tail of larger peak
- All peaks tail
  - Extracolumn effects
  - Build up of contamination on column inlet
  - Heavy metals
  - Column has aged and gone “bad”

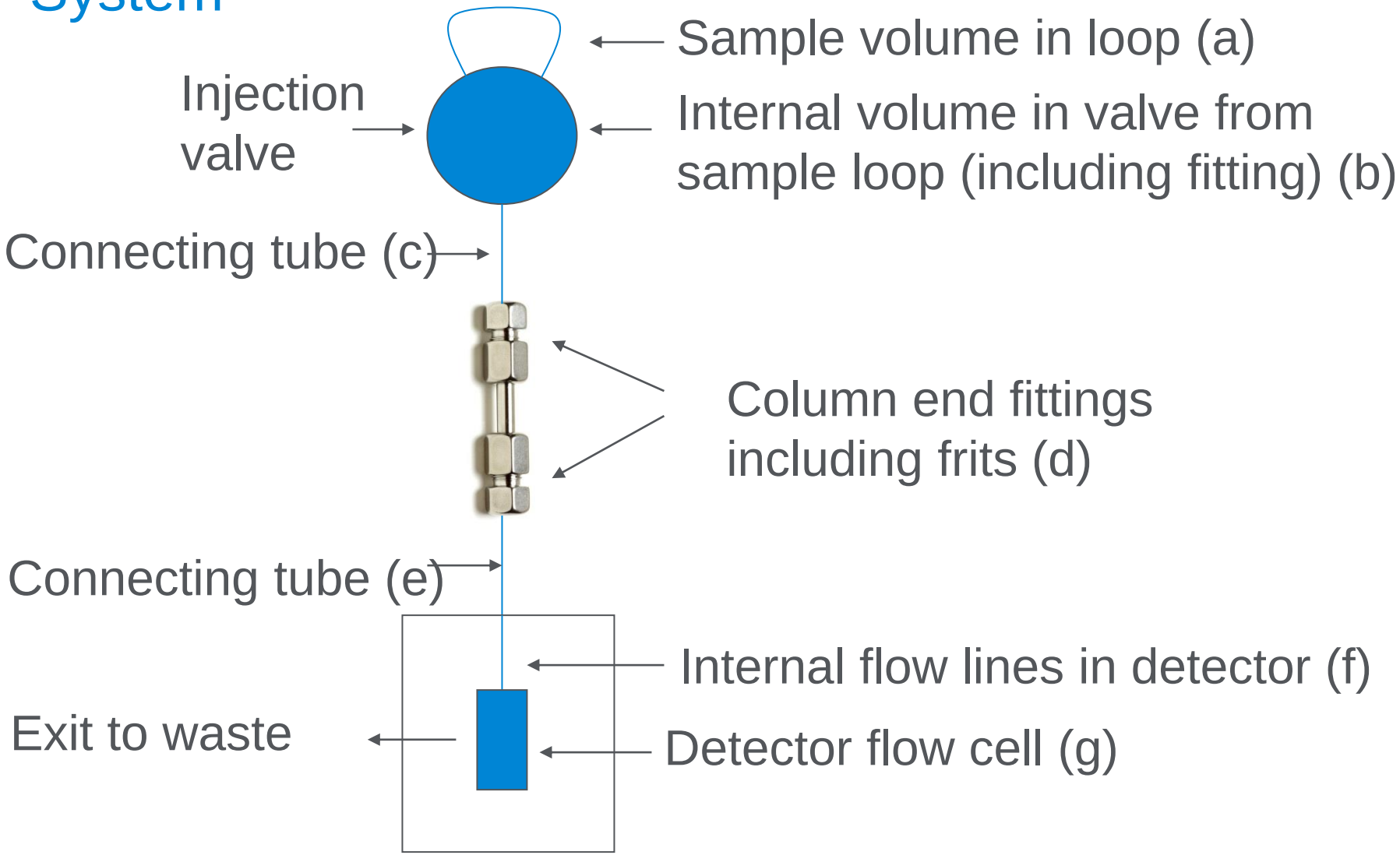
Extracolumn Dispersion (Volume)



Increasing extracolumn volume

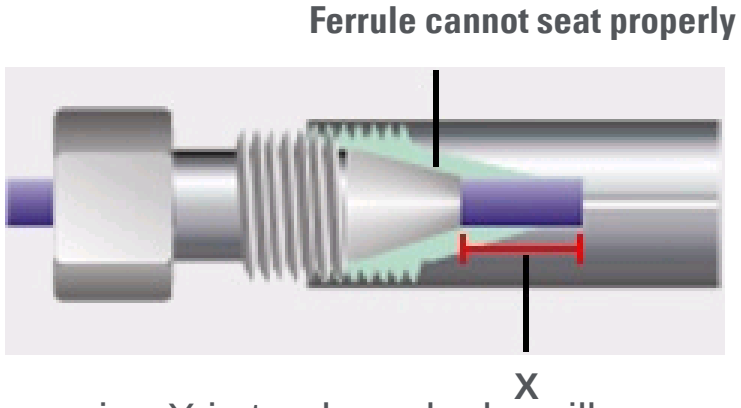
- Use short, small internal diameter tubing between the injector and the column and between the column and the detector.
- Make certain all tubing connections are made with matched fittings.
- Use a low-volume detector cell.
- Inject small sample volumes.

Extracolumn Volumes in HPLC Sample Flow System



What Happens if the Connections Are Poorly Made?

Wrong … too long

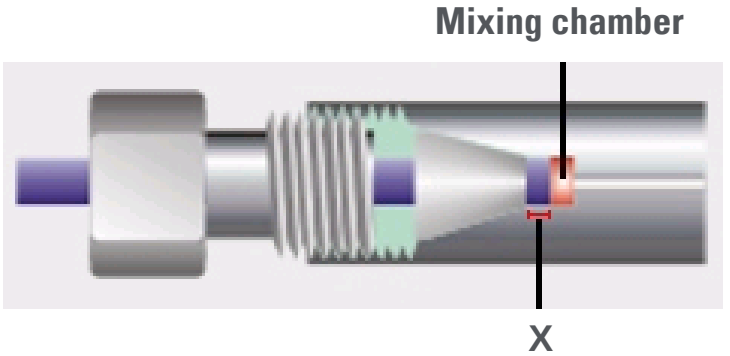


If dimension X is too long, leaks will occur.

These poor connections cause:

- Poor efficiency
- Peak tailing
- Leaking

Wrong … too short



If dimension X is too short, a dead volume or mixing chamber will occur.

Typical HPLC Problems

- 1/3 of problems are due to instrumental issues:
  - External leaks
  - Internal leaks: Pump seals, inlet and outlet valves
  - Injector maintenance: Rotor seal, needle seat, etc.
  - Poor connections
  - Data system not optimized

- 1/3 of problems are due to column issues:
  - Plugging, increasing pressure
  - Loss of bonded phase
  - Voids, settling

- 1/3 of problems are due to method problems:
  - Mobile phase incorrect (e.g. wrong pH, buffer concentration, solvent)
  - Inadequate sample preparation
  - Borderline ruggedness

Tools of the Trade

What should I keep in my lab?

- PM kits and spare parts
- Tubing and fittings
- Tools and testing supplies

PM Kits and Supplies

- Pump pm kit
- ALS pm kit
- Spare UV lamp
- Nebulizer rebuild
- Cleaning supplies for MS source
- Spare ion transfer capillary

AIV cartridge, 600 bar, G1312-60020

Tubing and Fittings

- Unions
- Swagelok nuts and ferrules
- PEEK tubing
- PEEK finger tight fittings
- PEEK tubing cutter and spare blades

ZDV universal union, 5022-2184

Union, female to female, 5042-8517

Tubing, PEEK, 0890-1762

Plastic tubing cutter, 8710-1930

Tools and Engineering Supplies

- Wrenches
- Phillips head screw driver
- Blanking plug
- Restriction capillary

Stainless steel blanking nut,
01080-83202

Blanking nut, stainless steel,
for M4 fittings ports, 5067-6141

Compact tool kit, G4296-68715

Troubleshooting

Issue statement: What specifically is wrong?

Make a list of what the causes might be.

How can I diagnose these causes?

What Specifically Is Wrong?

Example of issue statements with increasing detail:

- Retention time is shifting.
- Retention time is shifting earlier.
- Retention time is shifting to earlier times and the extent of the shift appears to be random.

Make a List of What the Causes Might Be

Using our previous example of retention time shifting:

- Changes to mobile phase composition
  - In the reservoir
  - Mixing
  - Pumping
- Interference or contamination
  - Contamination in flow path?
  - Ghost peaks?
- Changes to bonded phase
  - Much more likely to cause consistent time changes rather than random

Benchmarking

- System
- Column
- Maintenance notebook

Benchmarking an HPLC System

- Measure delay volume, record this value in the system maintenance logbook. Record the delay volume on the pump with a piece of tape, for quick reference.

- Inject uracil and naphthalene (assuming C18 and common checkout standard). Compare to LC Column Performance Report. Differences in dead time (uracil) and peak statistics (naphthalene) indicate differences in post column volumes.

LC Column Performance Report

Agilent Technologies

SERIAL NUMBER: USFHB03977

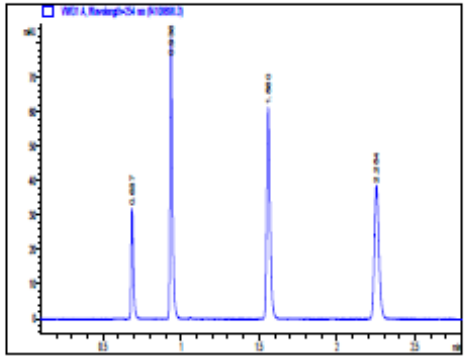
PART NUMBER: 653750-902
COLUMN TYPE: AdvanceBio Peptide Map 2.1 x 150 mm, 2.7 µm
PACKING LOT #: B20417

TEST CONDITIONS

MOBILE PHASE	=	70% Acetonitrile / 30% Water
COLUMN PRESSURE	=	307.3 Bar
COLUMN FLOW	=	0.40 ml / min
LINEAR VELOCITY	=	0.364 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	1 µl

QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE

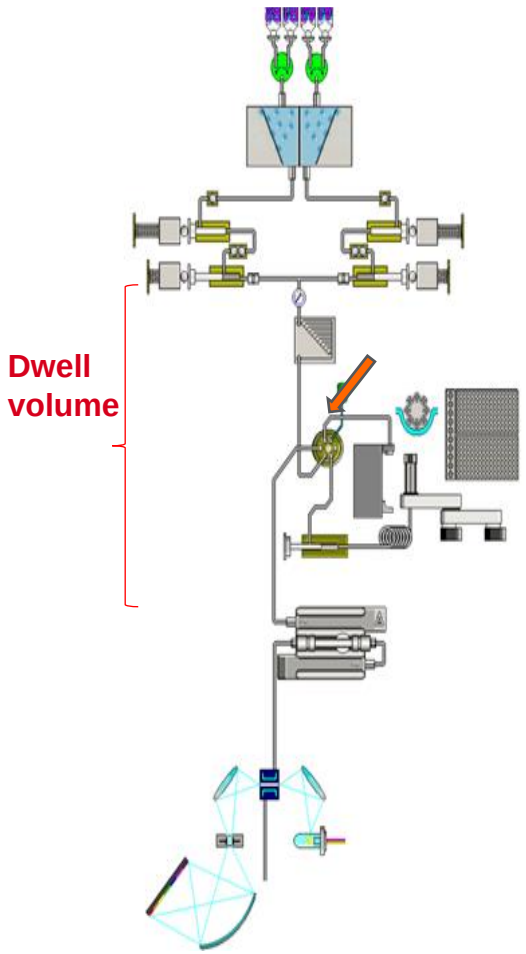
	TEST VALUES	SPECIFICATIONS
THEORETICAL PLATES =	29907	MIN = 24000
SELECTIVITY =	1.80	RANGE = 1.78 - 1.88
USP TAILING FACTOR = (@ 5% Peak Height)	1.19	RANGE = 0.98 - 1.20
k' =	2.28	



Sample components with concentrations diluted in mobile phase in the following elution order.

Peak #	Conc (ug/ml)	Sample Component
1	10	Uracil
2	400	Phenol
3	50	4-Chloro Nitrobenzene
4	80	Naphthalene

Determining the Dwell Volume of Your System



Look it up in the LC manual or follow the procedure below:

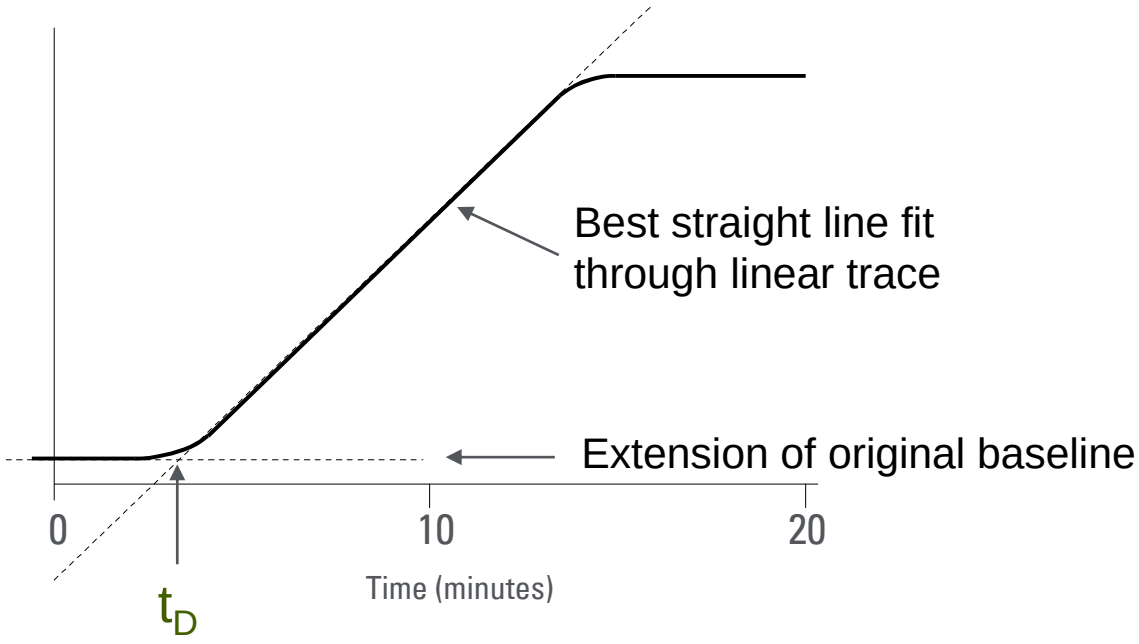
1. Replace column with a short piece of HPLC stainless steel tubing.
2. Prepare mobile phase components.

   A. Water – UV-transparent.
   B. Water with 0.2% acetone – UV-absorbing.
3. Monitor at 265 nm.
4. Run gradient profile 0 to 100% B/10 min at 1.0 mL/min.
5. Record – extrapolate best straight line fit through linear trace to baseline. Intersects at delay time.
6. Calculate delay volume from flow rate and delay time.

Expected dwell volume in UHPLCs – µL range!

Measuring Dwell Volume (V_D)

$V_D = t_D \times F$



- Intersection of the two lines identifies dwell time (tD).
- Dwell volume is equal to product of the flow rate and the dwell time.

Benchmarking a Column

In order to continually assess column performance, without any extra sample prep and injections, consider benchmarking a new column. Use either a control sample or an internal standard. If you use both in your methods, include both in your benchmarking.

- Prepare six sample vials at the same concentration that is used during routine analysis.
- Inject once per vial.
- Create a statistical quality control (SQC) chart: Plot in Excel and make note of average and standard deviation.
- When running analysis compare control sample results to SQC chart. Add passing control results from analytical sequences.

Maintenance Notebook and Record Keeping

Wise use of an SQC chart along with good maintenance records can often decrease the need to troubleshoot large issues by finding them before they occur.

- Keep a daily log of performance checks that were run along with any observations.
- Record all preventative maintenance (PM) as well as any repairs. Always follow any maintenance with a performance check.
- Maintain a tools and repair parts inventory sheet.
- Construct and use an SPQ chart.
  - Potential action thresholds could:
    - Result outside of control limits. Typically ± 3 standard deviations.
    - Be trending data, e.g. five to seven consecutive points above or below average.

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

Available in the USA & Canada 8 a.m. to 5 p.m. all time zones

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

Questions?